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GRANT NO: DAMD17-94-J-4182

TITLE: IN VIVO TRANSCRIPTIONAL REGULATION OF THE HUMAN HMG-1/Y GENE AS IT RELATES TO THE PROGRESSION OF BREAST CANCER

PRINCIPAL INVESTIGATOR:

Dr. Laurel S. T. Holth

CONTRACTING ORGANIZATION:

Washington State University
Pullman, Washington 99164-3140

REPORT DATE:

August 23, 1995

TYPE OF REPORT:

Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	23-Aug-95	Final 8 Sep 94 - 31 Jul 95

4. TITLE AND SUBTITLE

IN VIVO TRANSCRIPTIONAL REGULATION OF THE HUMAN HMG-1/Y GENE AS IT RELATES TO THE PROGRESSION OF BREAST CANCER

5. FUNDING NUMBERS

DAMD17-94-J-4182

6. AUTHOR(S)

Dr. Laurel S. T. Holth

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Washington State University
Pullman, Washington 99164-3140

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Reasons for early termination of award is that author received Ph.D. in June 1995

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Increased HMG (high mobility group)-I/Y expression is correlated with more advanced cancers and with high metastatic potential. To determine what regulates the human HMG-I/Y gene in breast cancer, we studied the expression of the HMG-I/Y gene in two human breast cancer cell lines: MCF7 cells which are estrogen receptor positive and Hs578T cells which are estrogen receptor negative. HMG-I/Y expression was studied in the presence and absence of β-estradiol and epidermal growth factor (EGF). No increase in HMG-I/Y messenger RNA was observed in β-estradiol or EGF treated MCF7 cells. In contrast, in the more metastatic Hs578T cell line there is a dramatic increase in HMG-I/Y mRNA expression, up to twenty-three fold, when treated with EGF. HMG-I/Y mRNA expression was further characterized in the EGF treated Hs578T cells using primer extensions, which showed that two of the multiple possible HMG-I/Y transcripts are induced. HMG-I/Y mRNA is very stable with a $t_{1/2}$ ~30 hours. Nuclear run-off transcription assays demonstrated an increase in the transcription rate of the HMG-I/Y gene in the presence of EGF. Western blots of protein extracted from Hs578T cells induced with EGF showed HMG-I/Y protein levels increased concurrently with the mRNA. The HMG-I/Y protein functions as an 'architectural transcription factor', thus the EGF induction of HMG-I/Y potentially provides a novel mechanism for a global alteration of gene expression in cancerous cells by a growth factor/kinase signalling pathway.

14. SUBJECT TERMS

HMG-I/Y
Breast Cancer

15. NUMBER OF PAGES

39

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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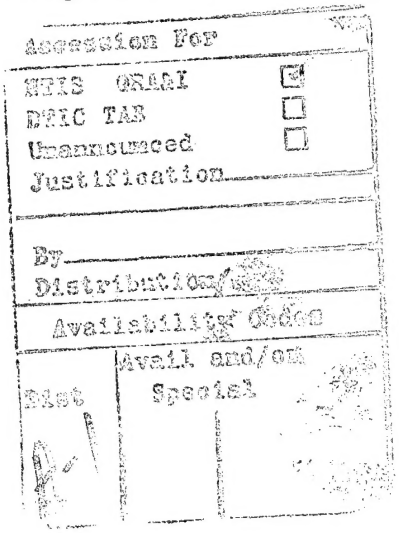

PI - Signature Aug. 23, 1995 Date

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Hormonal Regulation of the Human HMG-I/Y Gene in Breast Cancer Cell Lines

L.S.T. Holth, A.E. Thorlacius and R. Reeves

Department of Genetics and Cell Biology, Washington State University, Pullman, WA 99164-4660.

Key Words: High Mobility Group Proteins; HMG-I/Y; EGF (epidermal growth factor): breast cancer; mathematical model

Abbreviations: bp, base pair; DTT, dithiothreitol; EGF, epidermal growth factor; GAPDH, glyderaldehyde-3-phosphate dehydrogenase, HMG, High Mobility Group nonhistone chromatin proteins; mRNA, messenger RNA; nt, nucleotide; TPA, 12-O-tetra-decanoyl phorbal 13 acetate; UTR, untranslated region.

NOTE: THE FOLLOWING REPORT (WITH SOME MODIFICATIONS) HAS BEEN SUBMITTED TO THE JOURNAL *ONCOGENE*, AND USED AS A CHAPTER IN MY (DR. LAUREL HOLTH) Ph.D THESIS AT WASHINGTON STATE UNIVERSITY (JUNE 1995).

INTRODUCTION

HMG-I and HMG-Y are high mobility group, non-histone chromatin proteins which bind the minor groove of A-T rich DNA (Elton *et al.,* 1987; Reeves *et al.,* 1987; Solomon *et al.,* 1986; Strauss *et al.,* 1984; Reeves *et al.,* 1990; Churchill *et al.,* 1991; Reeves *et al.,* 1993). They are encoded by the same gene, and produced by alternate splicing (Friedmann *et al.,* 1993; Johnson *et al.,* 1989). HMG-I contains 11 internal amino acids that are absent in HMG-Y; the functional difference of the two proteins is not known (Johnson *et al.,* 1989). HMG-I/Y regulates *in vivo* gene transcription in either a positive (e.g. murine lymphotoxin (Fashena *et al.,* 1992), interferon-β (Thanos *et al.,* 1992; Du *et al.,* 1993), E-Selectin (Lewis *et al.,* 1994; Whitley *et al.,* 1994), human IL-α (John *et al.,* 1995), rat rRNA (Yang-Yen *et al.,* 1988)) or a negative (e.g. murine IL-4 (Chuvpilo *et al.,* 1993), GP91-PHOX (Skalnik *et al.,* 1992), murine ε-IgG (Kim *et al.,* 1995)) manner. HMG-I/Y is not a traditional gene regulatory factor, but rather represents a new class of structural/regulatory proteins, "architectural transcription factors". HMG-I/Y can bend and change the conformation of DNA (Nissen *et al.,* 1995), to facilitate the formation of active multiprotein transcription regulatory complexes (Fashena *et al.,* 1992; Thanos *et al.,* 1992; Du *et al.,* 1993; John *et al.,* 1995). For example, HMG-I/Y interacts directly with sequence-recognizing activator proteins (e.g. NFκ-B, ATF-2, NFAT, or ELF-1) and affects their DNA-binding affinities (e.g. NFκ-B, ATF-2, NFAT)(Fashena *et al.,* 1992; Thanos *et al.,* 1992; Zhoa *et al.,* 1993). HMG-I/Y has also been proposed to be involved in the formation of active chromatin: HMG-I/Y mobilizes histone H1 at or near scaffold attachment regions, and the subsequent H1 redistribution in the adjacent regions is postulated to facilitate the binding of factors involved in the displacement of nucleosomes and potentiate binding of transcription factors (Zhoa *et al.,* 1993). In this context, the increased levels of HMG-I/Y associated with increased metastatic potential of cancerous cells may lead to inappropriate formation of active chromatin resulting in aberrant gene expression.

Most normal, differentiated mammalian cells express low levels of HMG-I/Y mRNA and protein (Johnson *et al.,* 1990; Elton *et al.,* 1986; Johnson *et al.,* 1988; Jyranoja *et al.,* 1991; Lundberg *et al.,* 1989; Ostvold *et al.,* 1985). In contrast, cancerous cells (Reeves *et al.,* 1987; Johnson *et al.,* 1990; Johnson *et al.,* 1988; Bussemakers *et al.,* 1991; Giancotti *et al.,* 1991; Giancotti *et al.,* 1989; Giancotti *et al.,* 1987; Lund *et al.,* 1985; Manfioletti *et al.,* 1991; Chiappetta *et al.,* 1995) and embryonic cells which have not yet differentiated (Vartiainene *et al.,* 1988) often express high levels of HMG-I/Y mRNA and protein. Spontaneously derived tumors, or normal cells experimentally transformed with chemicals, ionizing radiation, UV, viral oncogenes (*v-src, v-ras, v-mos, v-myc*) all contain abnormally high levels of HMG-I/Y mRNA and protein (Elton *et al.,* 1986; Johnson *et al.,* 1988; Bussemakers *et al.,* 1991; Giancotti *et al.,* 1991; Giancotti *et al.,* 1989; Giancotti *et al.,* 1987; Manfioletti *et al.,* 1991; Ram *et al.,* 1993; Chiappetta *et al.,* 1995). Not only does cellular transformation and loss of a differentiated phenotype result in increased expression of HMG-I/Y, but the reverse is also true; in undifferentiated, highly aggressive tumor cells induced to undergo differentiation HMG-I/Y levels drop, and *in vivo* tumorigenic potential is lost (Vartiainene *et al.,* 1988). The extent of HMG-I/Y over-expression in a well characterized mouse mammary epithelial cell system (Ram *et al.,* 1993), the Dunning

rat model system for prostate cancer (Bussemakers *et al.,* 1991) and in human thyroid neoplasias (Chiappetta *et al.,* 1995) directly correlates with degree of metastatic aggressiveness of the tumor and not with growth rate (Bussemakers *et al.,* 1991; Ram *et al.,* 1993; Chiappetta *et al.,* 1995). More importantly, in a retrospective study of prostate cancer tumors from 71 patients which assessed HMG-I/Y mRNA expression by *in situ* hybridization, it was observed that HMG-I/Y expression is related to both the state of tumor differentiation and to Gleason grade in prostate cancer (Tamimi *et al.,* 1993). Similarly, a correlation has recently been found between HMG-I and HMG-Y expression and the malignant phenotype of thyroid neoplasias (Chiappetta *et al.,* 1995). Furthermore, it has been recently demonstrated that inhibition of HMG-I/Y protein synthesis by gene antisense methodology suppresses the ability of transforming retroviruses to induce neoplastic transformation in rat thyroid cells (Berlingieri *et al.,* 1995). Therefore, understanding the changes in regulation of HMG-I/Y when going from a normal differentiated cell type to a transformed undifferentiated cell type should provide fundamental insights into the changes which occur in cells when they become cancerous.

As has been outlined above, increased HMG-I/Y expression is correlated with more advanced cancers and with high metastatic potential; this correlation is also true for the epidermal growth factor receptor (EGF-R) for breast cancer. A plethora of reports link high EGF-R levels with poor clinical prognosis (Klijn *et al.,* 1992). Patients with advanced breast cancer with EGF-R positive tumors respond less well to first-line endocrine treatment as compared to EGF-R negative tumors (Klijn *et al.,* 1992). Understanding the downstream cellular effects of EGF (and the other ligands for the EGF receptor) on tumor cells which express the EGF-R but which do not exhibit a proliferative response to EGF, may lead to important insights into the role of the EGF receptor in cancers which have poor tumor differentiation and grade .

It was therefore of interest to determine the effect of EGF on HMG-I/Y expression in several human breast cancer cell lines. Hs578T cells are a human mammary tumor-derived line of infiltrating ductal carcinoma cells of epithelial origin (Hackett *et al.,* 1977). The malignant Hs578T cells have high levels of EGF-R, but do not require EGF to grow (Ennis *et al.,* 1989; Li *et al.,* 1992), and do not exhibit a proliferative response to EGF (Ennis *et al.,* 1989; Li *et al.,* 1992). In addition, Hs578T cells are estrogen receptor negative (Hackett *et al.,* 1977). In contrast, MCF7 cells are a human breast adenocarcinoma cell line that is estrogen receptor positive (Thompson *et al.,* 1992). MCF7 cells form tumors in nude mice, but unlike Hs578T cells MCF7 cells are not metastatic (Thompson *et al.,* 1992). Both Hs578T and MCF7 cells are derived from epithelial cells (Hackett *et al.,* 1977; Thompson *et al.,* 1992). Here we report that EGF induces HMG-I/Y message up to 23 fold in metastatic Hs567T cells, but no induction of HMG-I/Y is observed in the non-metastatic MCF7 cells. This is an important observation as EGF induction of HMG-I/Y potentially provides a global mechanism for alteration of gene expression in cancerous cells through a growth factor/ kinase signalling pathway.

The human HMG-I/Y gene has an extremely complex structure (Friedmann *et al.,* 1993), (see figure 1), which is markedly different from genes coding for other families of HMG proteins (Johnson *et al.,* 1989). A multitude of different mRNAs can be produced from this gene by utilizing different transcriptional start sites and alternative splicing (Friedmann *et al.,* 1993). The four known transcription initiation sites are located at the

beginning of exons I, II, III, and V (see figure 1). The promoter region 5' to exon II is induced by phorbal esters (e.g. TPA) in K562 cells (Ogram *et al.,* 1995). This induction is mediated by the AP1-like transcription factor (Ogram *et al.,* 1995). Here we report that transcription starting at both exon I and exon II can be induced in metastatic cancerous human Hs578T breast epithelial cells by treatment with epidermal growth factor (EGF). In contrast, no induction of HMG-I/Y was observed in non-metastatic breast cancer epithelial MCF7 cells treated with EGF. This work, in combination with that of Ogram and Reeves (Ogram *et al.,* 1995), provides direct experimental support for the hypothesis that the transcripts produced from the HMG-I/Y gene are independently regulated, and that this regulation is dependent on both the cell type and the inducer. In this paper we also documented for the first time that HMG-I/Y protein levels increase concomitantly with messenger RNA levels in EGF stimulated cells.

BODY

Materials and Methods

Cell culture, RNA isolation and Northern hybridization

The human breast ductal carcinoma cell line Hs578T (American Type Culture Collection: ATCC HTB 126) was maintained in DMEM medium (Sigma) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA) and antibiotics (100 µg/ml each of penicillin-G and streptomycin obtained from Sigma Co., St. Louis, MO). The human breast adenocarcinoma cell line MCF7 (ATCC HTB 22) was maintained in DMEM medium without phenol red (Sigma) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), bovine insulin 10 µg/ml, and antibiotics (100 µg/ml each of penicillin-G and streptomycin obtained from Sigma Co., St. Louis, MO). Charcoal stripped serum was used for experiments looking at the effect of β-estradiol. Cells were stimulated as indicated for different times with 30 ng/ml of murine natural epidermal growth factor (EGF) (GIBCO-BRL), or 100 nM β-estradiol (Sigma), or TPA 96 ng/ml. Cycloheximide (Sigma) was added at a concentration of 10 µg/ml where indicated to determine if protein synthesis was required for gene induction. Actinomycin D (Sigma) was added to 10 µg/ml where indicated to determine the stability of HMG-I/Y and *c-myc* messenger RNA's. Cells were harvested at the indicated times after stimulation, and RNA was isolated using the guanidine isothiocyanate/cesium chloride method (Ausubel *et al.,* 1988). For each sample 20 µg of total RNA was used for Northern hybridization analysis as previously described (Wingett *et al.,* 1991). The probe used for HMG-I/Y mRNA was the cDNA clone 7C (Johnson *et al.,* 1989). The probe used for c-myc mRNA was the *c-myc* cDNA (ATCC). Equivalent loading of RNA was determined by detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the cDNA obtained from ATCC. Quantitation of autoradiographs was determined by densitometric analysis using a LKB XL Laser Densitometer (LKB AB, Sweden).

Primer extension analysis

Twenty µg of total RNA isolated as described above was used for primer extension analysis. About 100 ng of a synthetic oligonucleotide 39-mer corresponding to the antisense strand of the HMG-I cDNA nucleotides #42-81 (Johnson *et al.,* 1989) was end labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase (GIBCO-BRL) (Ausubel *et al.,* 1988). About $5x10^5$ cpm of labeled primer was used for hybridization analysis as previously described (Johnson *et al.,* 1989).

Western Blots

Hs578T cells (~3.5 x 10^6 cells/time point) were stimulated with 30 ng/ml of epidermal growth factor for times indicate, cells were then harvested and histone H1 and HMG (high mobility group) proteins were acid extracted (Reeves *et al.,* 1990). Acid extracted proteins were then electrophoresed on a 15% polyacrylamide gel and transferred to Immobulon-P membrane (Millipore) by electrotransfer, overnight at 14 volts in 20% methanol; 150 mM glycine; and 20 mM Tris, pH 8.3. Immunodetection of HMG-I and HMG-Y was performed using polyclonal anti-HMG-I (MR18) antibody produced in rabbit (Disney *et al.,* 1989). Detection was performed using the ECL Western Blotting System (Amersham). The anti-HMG-I antibody used did not discriminate between HMG-I and HMG-Y. The anti-HMG-I antibody had low cross reactivity with histone H1 which made it possible to normalize results to H1 levels. Quantitation of autoradiographs was determined by densitometric analysis using a LKB XL Laser Densitometer (LKB AB, Sweden).

Nuclear Run-off Transcription Assays

Nuclei were isolated from Hs578T cells which were harvested by washing in 1X PBS, trypsinizing, suspending in cold DMEM + 10% FBS, pelleting cells and resuspending in cold .75X PBS for 10 minutes to swell cells. Cells were then pelleted and resuspended in 4 ml sucrose buffer I (0.32 M sucrose; 3 mM $CaCl_2$; 2 mM magnesium acetate; 0.1 mM EDTA; 10 mM Tris-Cl, pH 8.0; 1 mM DTT; 0.5% Nonidet P-40) (Ausubel *et al.,* 1988). Nuclei were isolated by dounce homogenizing cells 4 times with a tight fitting pestle. Eight ml of sucrose buffer II (2 M sucrose; 5 mM magnesium acetate; 0.1 mM EDTA; 10 mM Tris-Cl, pH 8.0; 1 mM DTT) (Ausubel *et al.,* 1988) was then added and nuclei were harvested by ultra centrifugation at 30,000 x g for 45 min, 4°C. Nuclei were resuspended in 100 µl of glycerol buffer (50 mM Tris-Cl, pH 8.3; 5 mM $MgCl_2$; 0.1 mM EDTA; 40% glycerol) (Merscher *et al.,* 1994). The nuclear run-off transcription assays were performed using a chemiluminescent detection system outlined in reference (Merscher *et al.,* 1994), with the following modifications. Briefly 5-10 x 10^7 nuclei (100 µl) were mixed with 100 µl of 2X reaction buffer (10 mM Tris-Cl, pH 8.0; 5 mM $MgCl_2$; 0.3 M KCl; 1 mM DTT; 0.2 mM EDTA); 1 µl each of 100 mM ATP, CTP, GTP (Pharmacia Biotech) and 4 µl of DIG-UTP (10 nM/µl, Boehringer Mannheim), incubated at 30°C for 30 minutes. Add 5µl DNase I, RNase free (10 u/µl, Boehringer Mannheim), and 10 µl $CaCl_2$ (20 mM), and incubate 5 minutes at 30°C. Add 10 µl Proteinase K, RNase free (10 mg/ml), 25 µl 10xSET (5% SDS; 50 mM EDTA; 100 mM Tris-Cl, pH 7.4), and 5 µl tRNA (5 mg/ml) and incubate 30 minutes at 42°C. Add 550 µl of NRO buffer II (4 M GITC; 25 mM Na Citrate, pH 7.0; 0.5% sarkosyl, 0.1 M 2-mercaptoethanol)(Merscher *et al.,* 1994), and 90 µl 2 M NaAc, pH 4.0. Extract twice with phenol-chloroform-isoamyl alcohol (24.5:24.5:1). Precipitate with 1 volume of isopropanol and precipitate -20°C, 20 minutes. Resuspend pellet in 300 µl NRO buffer II and 30 µl 2 M NaAc, pH 4.0 and reprecipitate with 825 µl EtOH. Wash pellet with 70% EtOH. Speed vac pellet dry and

resuspend in 200 µl 0.05% SDS. Hybridization and chemiluminescent detection were done as described in reference (Merscher *et al.*, 1994).

Mathematical model relating mRNA stability and transcription rate

Traditionally the half life of specific mRNAs is determined by inhibiting transcription using a transcriptional inhibitor such as actinomycin D. Alternatively the stability of a particular species of message can be determined by pulse chase. Both of these methods can present practical difficulties. The use of transcriptional inhibitors results in cell death and so is not useful for messages with long half lives. In pulse chase experiments it is difficult to detect transcripts which are not extremely abundant. Thus we have found the following to be a useful alternative, or supplement to the above techniques.

In the absence of any induction the amount of a particular mRNA at steady state will be determined by the rate of it's transcription and the half life of the mRNA in question. When expression of a particular message is induced there are three possible things which can occur: 1) the rate of transcription increases, 2) the stability of the message increases or, 3) both 1 and 2 occur. Thus there are basically three variables involved: 1) the initial message stability, 2) the final message stability, and 3) the change in transcription rate. Knowledge of any of these three can be used to solve for the other two using the equation which follows. If nothing is known about any of these three variables it is still possible to solve for the best fit for the data.

This equation determines the situation at any given time t and is used in a math program such as EXCEL 5.0 to solve over a continuous time spectrum. The variables are:
Ya = the initial stable quantity of message
Xa = the initial stability of the message in hours
Pa = the initial transcription rate of the message in relative units
Ra = the initial rate of transcript survival per hour
Xa can be converted to Ra using the equation $1/2 = Ra^{Xa}$, assuming exponential decay of the message.
Ya, Pa, and Ra can be related by the following equation, (where h is in the units of hours)
$Ya(1-Ra^{h}) = Pa \cdot h$, for very small h, because in stable state the amount that decays, i.e. $Ya(1-Ra^{h})$ must exactly be replaced by the amount produced, i.e. $Pa \cdot h$.
Solving for the limit of $h \rightarrow 0$, we find,
$Pa = -(\ln Ra)Ya$.
With the introduction of an inducer at time 0, the half life is now Xb, the rate of transcription is Pb and the half life is changed to Xb, then assuming exponential decay continues but at the new rate Rb where $1/2 = Rb^{Xb}$, then if Y(t) is the level of message occurring at time t we have,
$hY'(t) = Y(t) - Y(t + h)$ for small h, by definition of Y'(t)
where $Y(t + h) = Y(t)Rb^{h} + Pb \cdot h$ for small h,
where $Y(t)Rb^{h}$ the amount of Y(t) left at Y(t+h) and $Pb \cdot h$ is the amount produced in time t, and the amount of decay of $Pb \cdot h$ is negligible as $h \rightarrow 0$.
Taking the limit we find,

$Y'(t) = (\ln Rb)Y(t) + Pb$, and solving for $Y(t)$, knowing $Y(0) = Ya$, we find, $Y(t) = Yb + (Ya - Yb)Rb^{-t}$ where $Ya = -Pb/\ln Ra$, $Yb = -Pb/\ln Rb$ and $dY/dt = Y'(t)$. It should be noted that the above equation has the following advantages: 1) it can be used for both cases where the inducer causes an increase in message and for cases where a decrease in message is observed because the inducer has been withdrawn. 2) unlike traditional methods of measuring stability where transcription of the message being studied must be stopped (either with a transcription inhibitor, or in the case of pulse-chase by removing the label), using this equation it is possible to use data from experiments where the levels of message change but transcription is still continuing. 3) the relative change in the transcription rate can be determined using this equation. We have found that this equation provides a close fit to all the data we have analyzed, for example see figure 6B and 6D.

Results

HMG-I/Y gene inducible by EGF in metastatic breast cancer cell line, but not in non-metastatic line

Here we report that EGF induces HMG-I/Y message up to 23 fold in metastatic Hs567T cells (see figure 2A), but that no induction of HMG-I/Y is observed in the non-metastatic MCF7 cells, (see figure 2B). No induction of HMG-I/Y was observed in either cell line when cells were induced with insulin (data not shown).

It has been previously reported (Lanahan *et al.,* 1992) that HMG-I/Y is a 'delayed early response' gene in BALB/MK mouse cells maintained in serum free media and induced to proliferate with FGF, PDGF or EGF and in CTLL cells induced to grow with IL-2. In order to determine if protein expression was required for the induction of HMG-I/Y by EGF in Hs578T cells, these cells were grown in media containing cycloheximide for four hours in either the presence or absence of EGF. A two fold induction in HMG-I/Y expression was observed in Hs578T cells treated with EGF and cycloheximide versus untreated cells, or cells treated only with cycloheximide, (see figure 2A). However the induction of HMG-I/Y message in cells treated with EGF and cycloheximide was not as high as the four fold induction in HMG-I/Y message observed in Hs578T cells treated for four hours with EGF alone, suggesting that a partial induction of HMG-I/Y message is possible in the absence of protein synthesis, but that protein synthesis is required for full induction of the HMG-I/Y gene by EGF. As a control, the same experiments were done with *c-myc*, as cycloheximide should give a super induction for this gene, which it did, (see figure 3A). To our knowledge, this is the first report of EGF inducing *c-myc* in Hs578T cells.

Estrogen has no effect on HMG-I/Y expression in MCF7 cells

As MCF7 cells are estrogen receptor positive, and estrogen is known to increase cellular proliferation and gene expression in these cell lines (Pratt *et al.,* 1993) it was of interest to determine if estrogen had an effect on HMG-I/Y expression. No significant induction of HMG-I/Y message was observed in MCF7 cells induced with β-estradiol, (see figure 2C). This result fits with the correlation of higher levels of HMG-I/Y being observed in more metastatic cancers, as estrogen receptor positive tumors are generally of low metastatic potential (Thompson *et al.,* 1993). As a control to show that the β-estradiol induction was working, northern blots were also probed for *c-myc* expression, (see figure 3B). A large induction in *c-myc* message was observed, as has been previously reported (Dubik and Shiu, 1992).

Two HMG-I/Y transcripts are specifically induced by EGF

As diagrammed in figure 1, the human HMG-I/Y gene is very complex and produces multiple transcripts by using alternate transcription start sites and alternative splicing. It has been hypothesized that the different transcription start sites of the HMG-I/Y gene will be independently regulated (Johnson *et al.,* 1989; Friedmann *et al.,* 1993) with some of the start sites being inducible, and the regulation of these transcription start sites being dependent on cell type and inducer (Ogram *et al.,* 1995). It was therefore of interest to determine which transcripts were being produced in both uninduced and EGF induced Hs578T and MCF7 cells. Primer extension analysis showed that the 1A and 2B/7C transcripts were the most abundant in both uninduced Hs578T cells and MCF7 cells, (see figure 4). The pattern of transcript production was similar not only between Hs578T cells and MCF7 cells but also to that previously observed for K562 cells (Johnson *et al.,* 1989; Ogram *et al.,* 1995). It has recently been reported (Ogram *et al.,* 1995) that the second transcription start site alone is induced by TPA in K562 cells. Here we report evidence from primer extension analysis that both transcription start site 1 and 2 are inducible by EGF in Hs578T cells, although to different degrees. This is the first report of transcription start site 1 being inducible, and the first report of more than one transcription start site being regulated in the HMG-I/Y gene. The induction of the 2B/7C messages (transcription start site 2), is ~ 1.5 fold greater than that of the 1A message (transcription start site 1). As expected, there was no difference observed in the pattern of message production in MCF7 cells in either the presence or absence of EGF as their was no EGF induction of HMG-I/Y in these cells.

HMG-I/Y mRNA is very stable

It has been shown previously that the HMG-I/Y mRNA is very stable ($t_{1/2} > 6$ hours) (Johnson *et al.,* 1990; Ogram *et al.,* 1995). To confirm this in Hs578T cells we treated

these cells with actinomycin D (10μg/ml) for the times indicated, (see figure 5A). No decrease in HMG-I/Y message was observed in either the presence or absence of EGF, indicating a $t_{1/2} > 6$ hours. The same experiment was done for *c-myc* ,which has a short half life, as a control, see figure 5B. We show here that the stability of the *c-myc* message increases in the presence of EGF, an observation previously reported by Dean *et al.*, 1986.

To determine, an approximate $t_{1/2}$ for the HMG-I/Y message the following two experiments were done. HMG-I/Y was induced with EGF in Hs578T cells for 86 hours, the cells were then extensively washed with 1X PBS (phosphate buffered saline) and fed with media lacking EGF. Cells were then harvested at various times after the removal of the EGF to follow the decay of the message, (see figure 6A). The same experiment was also done using Hs578T cells induced with TPA (96 ng/ml) for 7 hours, (see figure 6B). Mathematical modeling was used to find a best fit for the data and to calculate the $t_{1/2}$ of the message (see figure 6A and B). Using this mathematical model we found for the experiment with EGF (see figure 6A) that HMG-I/Y has a $t_{1/2}$ of ~28 hours, and for the experiment with TPA (see figure 6B) that HMG-I/Y has a $t_{1/2}$ of ~31.5 hours. The variation in the two half life's is likely due to variation between the time it takes for the effect of the TPA versus the EGF induction to be removed from the cells. The same equation can be used to look at the induction of the message with EGF (see figure 2A). Knowing that the message is very stable with a half life of approximately 28 hours, we can use the above equation to calculate that the change in the transcription rate is approximately 23 fold assuming the stability of the message does not vary. Furthermore, due to the long half life of the HMG-I/Y messages it would be impossible to account for the increase in HMG-I/Y over the time course observed without a large change in the transcription rate, since to obtain the increase in HMG-I/Y message observed in the time indicated would be impossible by increasing message stability alone, even if the initial half life was as short as 6 hours.

Nuclear run-on transcription assays

Nuclear run-on transcription assays were done on Hs578T cells that were either uninduced or induced with EGF for four hours immediately prior to harvesting of the cells, (see figure 7). Two probes were used for the HMG-I/Y gene, probe A (nt# -177 to +2322, see figure 1) which contains both transcription start sites 1 and 2 which were shown by primer extensions to be inducible by EGF, and transcription start site 3 which is not observed in the primer extensions; and probe B (nt# +2323 to +6588, see figure 1) which contains transcription start site 4 and most of the protein coding region of the gene. Probe A, showed and increased signal for Hs578T cells induced with EGF over the uninduced cells, (see figure 7), although the increase observed was not as great as would be expected from the *in vivo* induction observed in the mRNA (see figure 2A). One possible explanation for this is that probe A contains two poly-T regions on the sense strand, (poly-A on the anti-sense strand). It has been previously reported that repetitive sequences, for example a poly-A tract in a cDNA probe, cause cross-hybridization on nuclear run-on transcription assays resulting in signals which appear constitutive (Brorson *et al.*, 1991). Probe B

shows no change when Hs578T cells are induced with EGF, this is probably due in part to cross-hybridization with the three poly-T regions found in this sequence. It is noteworthy that runs of T residues and a G-C rich stem loop structure are associated with premature termination in eukaryotic cells (Krumm *et al.,* 1993). G-C rich sequence which could form stem loop structures are found near all the poly-T stretches in the HMG-I/Y gene. It therefore seems plausible that premature termination of transcripts initiating in the promoter region of probe B (e.g. at transcription start site 4 or an unknown transcription start site) is occurring in uninduced Hs578T cells, while cells induced with EGF have increased transcriptional activity at transcription start sites 1 and 2 and these transcripts are fully elongated. It has been reported that products of premature termination generally have extremely short half lives and are therefore not detectable using northern blots or primer extensions (Krumm *et al.,* 1993).

Translation parallels increase in HMG-I/Y transcripts

Western detection was use to determine the relative levels of HMG-I/Y protein at the times indicated after the addition of EGF in Hs578T cells (figure 8). Results were normalized to the levels of histone H1, as the HMG-I/Y polyclonal antibody used had some minor cross reactivity to histone H1 (not shown). The increased levels of HMG-I/Y mRNA observed in Hs578T cells when induced by EGF are paralleled by increased levels of HMG-I/Y protein, see figure 8. Significant increases in HMG-I and HMG-Y protein levels are first observed eight hours after induction with EGF in Hs578T cells, with the levels of HMG-I/Y protein leveling off at 72 hours. Two bands can be seen for each time point in figure 8, the top band is HMG-I and the bottom band is HMG-Y. A much greater increase is observed in HMG-I levels (~14 fold at 72 hours +EGF) than in HMG-Y levels (~4 fold at 72 hours +EGF). The increases in the amount of HMG-I and HMG-Y are consistent with the primer extension results (see figure 4). The primer extensions in figure 4 show that the less abundant message is produced starting at transcription start site 1, this start site has only been observed to produce messages coding for HMG-Y (Johnson *et al.,* 1989). The second and more productive transcription start site can produce either HMG-I (transcript 7C) or HMG-Y (transcript 2B) (see figure 1). Since the western blots demonstrate that HMG-I protein is more abundant than HMG-Y protein it is probable that the second transcription start site is producing primarily the 7C message. These results clearly demonstrate that in these cells the level of HMG-I/Y protein is dependent on the level of HMG-I/Y message produced in Hs578T cells.

Uninduced Hs578T cells and MCF7 cells have similar levels of HMG-I/Y message, however relative to histone H1 levels the level of HMG-I/Y protein is much higher in Hs578T cells than in MCF7 cells (data not shown). Assuming that the HMG-I and HMG-Y proteins are equally stable in both cell types, this result suggests that the efficiency of translation of HMG-I/Y transcripts is greater in the more metastatic Hs578T cells than in MCF7 cells. In summary, HMG-I/Y does not appear to be translationally regulated within a single cell line (i.e. after EGF induction in Hs578T cells), but there does appear to be

variations in the level of translation of HMG-I/Y messages between different cell lines (i.e. between Hs578T cells and MCF7 cells).

DISCUSSION

Hormonal regulation of the HMG-I/Y gene in breast cancer

Two major growth-factor signaling cascades are involved in the normal growth regulation of epithelial cells (Aaronson *et al.,* 1990). One is activated by high concentrations of insulin, and the other by epidermal growth factor (EGF), transforming growth factor type-alpha (TGF-α), and members of the fibroblast growth factor family (FGF) (Aaronson *et al.,* 1990). EGF, TGF-α and amphiregulin (AR), are all ligands of the epidermal growth factor receptor (EGF-R), also known as erbB or erbB-1 (Aaronson *et al.,* 1990; Goldman *et al.,* 1990). Another oncogene, erbB-2, encodes a receptor protein which has a high degree of homology to EGF-R (Aaronson *et al.,* 1990). The erbB/EGF-R and erbB-2 genes are often amplified or over expressed in epithelial malignancies, particularly in breast cancers (Aaronson *et al.,* 1990; Klijn *et al.,* 1992). A plethora of reports link high EGF-R levels with poor tumor differentiation and grade and poor clinical prognosis (Klijn *et al.,* 1992). Patients with advanced breast cancer with EGF-R positive tumors respond less well to first-line endocrine treatment as compared to EGF-R negative tumors (Klijn *et al.,* 1992). There is also a relationship between erbB-2 over expression and poor overall survival in human breast cancer (Lupu *et al.,* 1993).

The role(s) of the EGF receptor system and erbB-2 in the oncogenic growth of cells is still poorly understood (Klijn *et al.,* 1992; Lupu *et al.,* 1993), but despite the evidence linking EGF-R levels with poor clinical prognosis, in most cases there does not appear to be a strong positive correlation between the over expression of EGF-R and higher rates of breast cancer proliferation (Klijn *et al.,* 1992). This suggests that the EGF-R over expression observed in many breast cancers may have a function in the oncogenic growth of cells which is separate and distinct from their role in cell proliferation. EGF and the other ligands for the EGF receptor are present in both normal and neoplastic human breast tissue. Understanding the downstream cellular effects of EGF (and the other ligands for the EGF receptor) on tumor cells which express the EGF-R but which do not exhibit a proliferative response to EGF, may lead to important insights into the role of the EGF receptor in cancers which have poor tumor differentiation and grade. In this context, it was interesting to observe that the HMG-I/Y gene was inducible by EGF in the metastatic Hs578T cells but not in the non-metastatic MCF7 cells.

There is an inverse correlation between the level of the EGF receptor and the estrogen receptor (ER) in breast cancer (Falette *et al.,* 1991; Toi *et al.,* 1991). While breast cancers which are ER-, with high EGF-R levels have poor prognosis and generally do not respond to chemotherapy (Toi *et al.,* 1991), ER+ tumors usually have not yet started to metastasize and are responsive to endocrine manipulation, and/or sensitive to cytotoxic

chemotherapy, and thus often have a good prognosis (Thompson *et al.,* 1993). It is therefore not surprising that since high level of HMG-I/Y expression are correlated with more advanced and metastatic cancers (Ram *et al.,* 1993; Bussemakers *et al.,* 1991; Tamimi *et al.,* 1993; Chiappetta *et al.,* 1995) that HMG-I/Y expression is not inducible by estrogen in MCF7 cells which are ER+ breast cancer cells (see figure 2C).

Regulation of the HMG-I/Y gene is very complex

The human HMG-I/Y gene is very complex with multiple levels of regulation. So far there is no evidence to suggest that the HMG-I/Y gene is regulated at the level of message stability, despite the production of multiple transcripts. This is not surprising as the HMG-I/Y message has a half life of approximately 28-31 hours (see Figure 6). However, the HMG-I/Y gene is regulated at the level of transcription and possibly by premature termination. Direct evidence for transcriptional regulation of the HMG-I/Y gene was first demonstrated with HMG-I/Y promoter-CAT constructs transfected into K562 cells induced with PMA (Ogram *et al.,* 1995). Here we show from the kinetics of increase of HMG-I/Y message, that since the HMG-I/Y message has such a long half life, the only way to account for the increase observed in HMG-I/Y message when induced by EGF is if transcription increases ~ 23 fold. An increase in the rate of transcription of the HMG-I/Y gene occurring in Hs578T cells induced with EGF was further confirmed using nuclear run-on transcription assays (see figure 7). However, the increase in transcription observed in the nuclear run-on transcription assays was not as great as expected from the increases observed with the northern blots. There are two possible complementary explanations for this. It has been reported that repetitive sequences, such as a poly(A) tract in a cDNA probe cause cross-hybridization in nuclear run-on transcription assays resulting in signals which appear constitutive (Brorson *et al.,* 1991). Unfortunately both fragments of the HMG-I/Y genomic clone that were used for probes on the nuclear run-on slot blot contained several long stretches of poly-T (sense strand) / poly-A (anti-sense strand), which conceivably could result in high levels of specific background and diminution in the differences observed between the EGF induced and the uninduced Hs578T cells. The poly-T regions observed in the HMG-I/Y gene (Friedmann *et al.,* 1993) are preceded by G-C rich regions which could form stem-loop structures and may function as sites for premature termination (Krumm *et al.,* 1993). It therefore seems possible that premature termination is being used as another mechanism for regulating the HMG-I/Y gene, however proof of this hypothesis will require further nuclear run on transcription experiments using an extensive series of probes that have the poly-T regions removed.

This paper together with that of Ogram and Reeves (Ogram *et al.,* 1995), provides direct experimental support for the hypothesis that differential regulation of the multiple transcription start sites in the HMG-I/Y gene depends both on the cell type and the inducer. The same inducer, EGF causes induction of HMG-I/Y in Hs578T cells, but not in MCF7 cells. However, the more interesting observation is that in K562 cells treated with TPA induction is only observed from transcription start site 2, although both transcription start sites 1 and 2 are being utilized in these cell (Ogram *et al.,* 1995). In contrast, in Hs578T cells induced with EGF both transcription start site 1 and 2 are

induced, although a greater induction is observed from start site 2. The HMG-I/Y gene is the first gene for which differential utilization and regulation of specific transcription start sites has been reported (Ogram *et al.,* 1995).

Both the 10A and 1A HMG-Y transcripts use transcription start site 1, however in primer extensions the 10A transcript has never been detected. This suggests that there is a strong splicing preference for the 1A transcript, or that the 10A transcript has a radically different and very short half life.

Complete induction of HMG-I/Y message requires protein synthesis, however there is a small but repeatable induction of HMG-I/Y mRNA by EGF in the presence of cycloheximide (see figure 2A). This suggests that to get full induction of HMG-I/Y message, protein factors that are required for this induction need to be synthesized, but that some of the factors needed for induction of transcription of HMG-I/Y are already present, prior to the addition of EGF, and in these cells are able to give a partial induction of the HMG-I/Y gene even in the absence of protein synthesis. It is possible that HMG-I/Y is involved in it's own regulation using a feedback loop to induce it's production, as there are several sites suitable for HMG-I/Y binding within the HMG-I/Y promoter regions (Friedmann *et al.,* 1993).

This publication is the first to show that HMG-I/Y protein levels increase with increasing message levels. However, despite similar levels of HMG-I/Y message in uninduced Hs578T cells and MCF7 cells, Hs578T cells express significantly more HMG-I/Y protein than MCF7 cells, suggesting that these different cell lines translate HMG-I/Y transcripts with different efficiencies.

The role of HMG-I/Y in cancer

The past several years have brought significant increases in our understanding of both the HMG-I/Y gene and protein. Based on this information the following scenario is proposed as a model for the role of HMG-I/Y in cancer. In this paper we have presented a biologically relevant mechanism by which increased levels of HMG-I/Y may be increased in increasingly metastatic breast cancers, namely by induction via the EGF receptor by EGF or other ligands of the EGF receptor such as amphiregulin and TGF-α. Furthermore, the correlation of increasing levels of HMG-I/Y with increased metastatic potential has also been specifically observed in prostate cancer (Bussemakers *et al.,* 1991; Tamimi *et al.,* 1993). In other types of cancer other mechanisms for increased levels of HMG-I/Y are likely varied, and it has been observed that cells transformed with transforming oncogenes (*v-src, v-ras, v-mos, v-myc*) have increased HMG-I/Y expression (Elton *et al.,* 1986; Johnson *et al.,* 1988; Bussemakers *et al.,* 1991; Giancotti *et al.,* 1991; Giancotti *et al.,* 1989; Giancotti *et al.,* 1987; Manfioletti *et al.,* 1991; Ram *et al.,* 1993; Berlingieri *et al.,* 1995). Furthermore, inhibition of HMG-I/Y protein synthesis by gene antisense methodology suppresses the ability of the *v-mos* and *v-ras*-Ki transforming retroviruses to induce neoplastic transformation (Berlingieri *et al.,* 1995). The human HMG-I/Y gene is located on the short arm of chromosome 6 at position 6p21 in a region in which numerous chromosomal abnormalities (translocations, rearrangements and amplifications) have been

observed in a variety of human cancers including leukemia, lymphoma and neuroblastoma (Friedmann *et al.,* 1993). Thus, there are several biologically relevant mechanisms which could lead to over expression of HMG-I/Y in cancer, and while HMG-I/Y is not a traditional oncogene, it is a potential point of convergence for the actions of many different pathways of oncogenic transformation.

From the complex regulation of the HMG-I/Y gene it can be postulated that the cell needs to carefully control the levels of HMG-I/Y and therefore that the consequences inappropriate levels of HMG-I/Y are potentially disastrous. HMG-I/Y has been demonstrated to regulate the expression of several genes by either increasing (e.g. murine lymphotoxin (Fashena *et al.,* 1992), interferon-β (Thanos *et al.,* 1992; Du *et al.,* 1993), E-Selectin (Lewis *et al.,* 1994; Whitley *et al.,* 1994), human IL-2Rα (John *et al.,* 1995), rat rRNA (Yang-Yen *et al.,* 1988)) or decreasing (e.g. murine IL-4 (Chuvpilo *et al.,* 1993), GP91-PHOX (Skalnik *et al.,* 1992), murine ε-IgG (Kim *et al.,* 1995))their rate of transcription. It is particularly noteworthy that HMG-I/Y has been shown to be involved in the regulation of the E-selectin gene which is expressed in endothelial cells, as E-selectin is a cellular adhesion molecule which has been implicated in tumor progression and metastasis (Banks *et al.,* 1993). HMG-I/Y is expressed at much higher levels then traditional transcription factors, and represents a new class of structural/regulatory proteins, "architectural transcription factors". HMG-I/Y can facilitate the formation of multiprotein transcription regulatory complexes (Fashena *et al.,* 1992; Thanos *et al.,* 1992; Du *et al.,* 1993; John *et al.,* 1995), and has been shown to affect the DNA-binding affinities of several sequence-recognizing proteins (e.g. NFκ-B, ATF-2, NFAT) (Fashena *et al.,* 1992; Thanos *et al.,* 1992; Zhoa *et al.,* 1993). This is likely due to the ability of HMG-I/Y to bend and change the conformation of DNA (Nissen *et al.,* 1995). It has also been proposed that HMG-I/Y is involved in the formation of active chromatin by displacing histone H1 at or near scaffold attachment regions (Zhoa *et al.,* 1993). As the HMG-I/Y protein recognizes the structure of DNA with a narrow minor groove, its binding is not limited to specific sequences (Elton *et al.,* 1987; Reeves *et al.,* 1987; Solomon *et al.,* 1986; Strauss *et al.,* 1984; Reeves *et al.,* 1990; Churchill *et al.,* 1991; Reeves *et al.,* 1993), and as the cellular levels of this protein are much greater than that of a traditional transcription factor (although they vary greatly depending on cell type), it seems highly probable that HMG-I/Y is involved in the regulation of a large percentage of genes, and is not limited to the eight genes mentioned above. Therefore, changes in the expression of HMG-I and HMG-Y could potentially lead to alterations gene expression and chromatin structure, thereby significantly contributing to the formation of the transformed phenotype.

Relationship between Reported Results and Original Research Proposal

The hypothesis of the original grant was as follows. The increased levels of HMG-I/Y mRNAs and protein, which are observed in increasingly metastatic cancerous cells are a result of a change in the transcriptional regulation of the HMG-I/Y gene at one or more of

its promoters. This hypothesis has been demonstrated to be correct, at least for the system studied in this manuscript.

This grant was held for 10 months, and was terminated prior to its expiration date of September 1996, as I was able to complete my Ph.D. in June of 1995. The first portion of the research proposal (RNA isolation, Northern blots, Primer Extensions) was completed, with modifications in the cell lines chosen and the addition of studying the effects of several different hormones, this work is reported here. The second part of the research plan (*in vivo* footprinting of the HMG-I/Y gene) was not completed, as it was in retrospect somewhat premature. There was not sufficient evidence prior to this study to know that the gene was being regulated at the level of transcription rather than message stability, or some other mechanism. Therefore, as reported here extensive message stability experiments and nuclear run-on transcription assays were done. The results of the nuclear transcription assays indicated that the rate transcription changes upon induction with EGF. However the data was somewhat confusing, and suggested the possibility of premature termination of transcription as yet another regulatory mechanism for this gene. Therefore, future studies of the HMG-I/Y gene should include more comprehensive studies of the HMG-I/Y gene with nuclear run-on transcription assays. Although not reported here, I also did studies of the HMG-I/Y promoter using promoter-CAT constructs. With the constructs I had, I did get promoter activity, however, it was not inducible with EGF, suggesting the DNA element responsible for EGF induction of the HMG-I/Y gene likely lay elsewhere, although a PMA response element was found in the promoter regions studied. Further characterization of the promoter of the HMG-I/Y gene with promoter-reporter constructs would be useful in pin-pointing areas to be studied using *in vivo* footprinting. Once further nuclear run-on transcription assays and promoter-reporter construct assays are completed, then it would be of interest to do *in vivo* footprinting studies as suggested in the original proposal, as these studies would still likely produce useful information and insights into the molecular mechanisms involved in the development and metastatic progression of breast cancer.

While, I believe continuation of this research is worthwhile, it is time for me to move on. As stated above, I completed my Ph.D. in June 1995. In July 1995, I began a post-doc in the lab of Dr. Jim Davie at the University of Manitoba in Canada. I will be studying the role of the nuclear matrix in estrogen receptor activity in human breast cancer, and will be submitting a new research proposal to the US Army on this work for the September 1995 deadline. This Army Graduate Student Research Fellowship has been an extremely valuable training experience for me. Having my own supply budget for the first time taught me useful lessons on the costs of research and running a lab, and gave me more control over my own work. The travel money allowed me to attend the American Cancer Association Annual Meeting in March 1995 to present this work. Attending meetings is a wonderful way to learn and meet other scientists, if it were not for this grant I likely would not have had this opportunity. I believe this is a useful program, and in my case has been successful, as I have completed graduate work and am continuing in breast cancer research as a post-doc.

Acknowledgments

We thank Sushma Ogram and Mark Nissen for many helpful discussions and technical assistance during the course of this work. In addition, we thank David Hill for help with figures. This work was supported by U.S. Army Breast Cancer Research Grant No. DAMD17-94-J-4182 to Laurel Holth.

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Figure Legends

Figure 1 Diagram of the human HMG-I/Y gene showing patterns of transcript initiation and alternative splicing. The human HMG-I/Y gene is greater the 10 kb in length and contains eight exons (Roman numerals I - VII) and seven introns (Arabic numerals 1 - 7). Nucleotide #1 indicates the start of exon I and arrows show putative transcription start sites that correspond to the beginning of previously cloned cDNAs coding for HMG-I/Y mRNAs. Solid lines connecting the various exons indicate different alternative splicing patterns that result in the production of all of the different cDNA clones isolated to date. BD-1, -2, -3 indicate the DNA-binding domains of the protein and the important protein coding and untranslated regions of the gene are designated by the shading key shown in the lower right hand corner of the diagram. A table of the different transcripts produced using different transcription start sites and alternative splicing is shown in the bottom left hand corner. Nucleotides -177, +2322, and +6588 are marked and deliminate the ends of probe A and B used in the nuclear run-off transcription assays.

Figure 2 Hormonal induction of expression of HMG-I/Y mRNA in breast cancer cells. Autoradiograms of RNA harvested from cells induced with either EGF or 17-β-estradiol for the times indicated (hours). Lanes marked - and + indicate cells grown in the presence of cycloheximide (10 μg/ml) for 4 hours in either the presence (+) or absence (-) of EGF (30 ng/ml). All lanes contain 20 μg of total cellular RNA. Each blot was probed with labeled HMG-I(7C) and GAPDH. Bar graphs show levels of HMG-I/Y detected, normalized to GAPDH. Panel A is Hs578T cells induced with EGF (30 ng/ml). Panel B is MCF7 cells induced with EGF (30 ng/ml). Panel C is MCF7 cells induced with 17-β-estradiol (100 nM).

Figure 3 Hormonal induction of expression of *c-myc* mRNA in breast cancer cells. Autoradiograms of RNA harvested from cells induced with either EGF or 17-β-estradiol for the times indicated (hours). Lanes marked - and + indicate cells grown in the presence of cycloheximide (10 μg/ml) for four hours in either the presence (+) or absence (-) of EGF (30 ng/ml). All lanes contain 20 μg of total cellular RNA. Each blot was probed with labeled *c-myc* and GAPDH. Bar graphs show levels of *c-myc* detected, normalized to GAPDH. Panel A is Hs578T cells induced with EGF (30 ng/ml). Panel B is MCF7 cells induced with 17-β-estradiol (100 nM).

Figure 4 Primer extensions of HMG-I/Y transcripts from total RNA isolated from breast cancer cell lines Hs578T and MCF7 induced with EGF (30 ng/ml) for times indicated in hours. Lanes marked - were not induced, lanes marked + were induced with EGF (30 ng/ml) for three days.

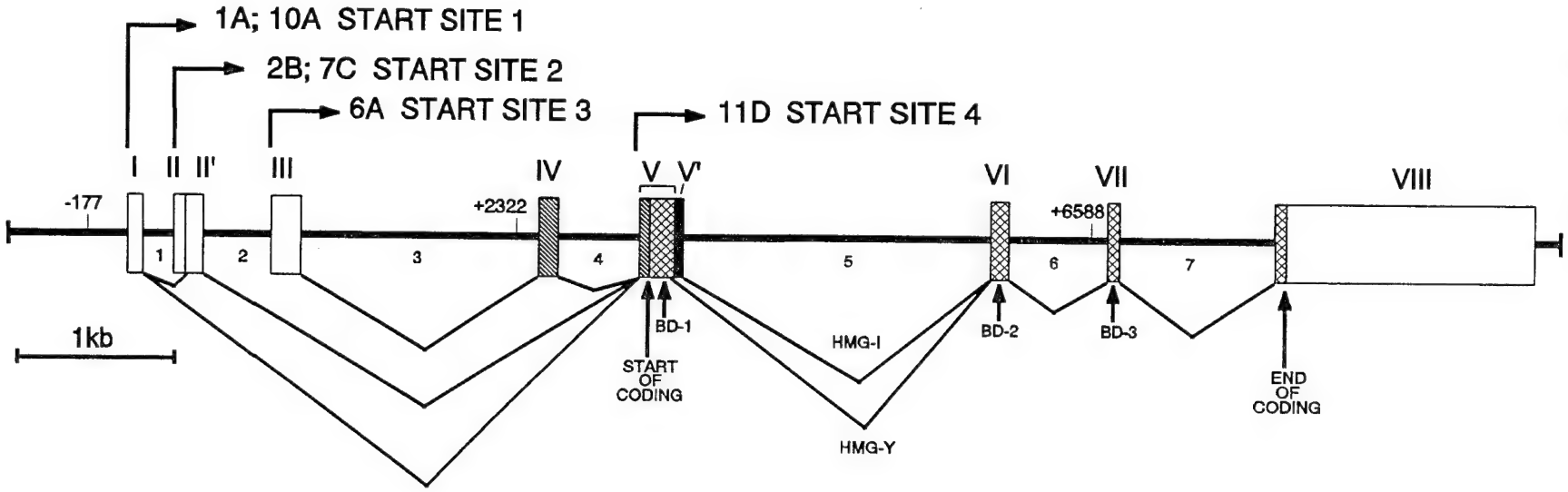
Figure 5 Actinomycin D was used to determine the stability of HMG-I/Y mRNA and *c-myc* RNA in Hs578T cells. Panel A shows cells harvested after exposure to actinomycin D (10 μg/ml) for times indicate in hours, where indicated cells were first treated with EGF (30 ng/ml) for 48 hours. Blots were probed with both labeled HMG-I/Y(7C) and GAPDH. Bar graph shows HMG-I/Y levels normalized to GAPDH. Panel B shows cells

harvested after exposure to actinomycin D (10 μg/ml) for times indicate in hours, where indicated cells were first treated with EGF (30 ng/ml) for 45 minutes. Blots were probed with both labeled *c-myc* and GAPDH. Bar graph shows *c-myc* levels normalized to GAPDH.

Figure 6 In order to determine the $t_{1/2}$ of the HMG-I/Y message, Hs578T cells were induced with either EGF or TPA and then washed twice with 1X phosphate buffered saline, and time points were harvested after the removal of the inducer, RNA was then harvested and 20 μg/lane were run on a Northern blot which was probed with both HMG-I(7C) and GAPDH. Panel A, lane 1, uninduced Hs578T cells; lane 2, Hs578T cells were induced with EGF (30 ng/ml) for 86 hours; lanes 3-7, Hs578T cells induced with EGF (30 ng/ml) for 86 hours, washed and then harvested 6, 12, 25, 36 and 52 hours later respectively. Panel B shows the data from panel A normalized to GAPDH and plotted -♦-. The data was modeled (shown as -□- line) using the equation detailed in the material and methods to determine a message half life of ~28 hours. Panel C, lane 1, uninduced Hs578T cells; lane 2, Hs578T cells induced with TPA (96 ng/ml) for 7 hours; lanes 3-7, Hs578T cells induced with TPA (96 ng/ml) for 7 hours, washed and then harvested 6, 18, 30, 42 and 50 hours later respectively. Panel D shows the data from panel C normalized to GAPDH and plotted -♦-. The data was modeled (shown as -□- line) using the equation detailed in the material and methods to determine a message half life of ~31.5 hours.

Figure 7 Nuclear run-on transcription assays done with Hs578T cells that were either unstimulated or induced with EGF (30 ng/ml) for 4 hours as indicated. Probe A contains the region of the HMG-I/Y gene extending from nt# -177 to nt# 2322. Probe B contains nt# 2323 to #6588 of the HMG-I/Y gene. The pBS-KS is shown as a negative control, the GAPDH is a positive control which is not induced by EGF.

Figure 8 Western blot of HMG-I and HMG-Y protein harvested from Hs578T cells induced with EGF (30 ng/ml) for times indicated in hours.



Alternatively Processed cDNA Clones

Clone	5'UTR	33-mer
11D	V	-
10A	I, V	-
1A	I, II', V	-
2B	II, II', V	-
7C	II, II', V	+
6A	III, IV, V	+

Untranslated cDNA

Untranslated ORF

Protein Coding

33 mer, I/Y splicing

FIGURE 1

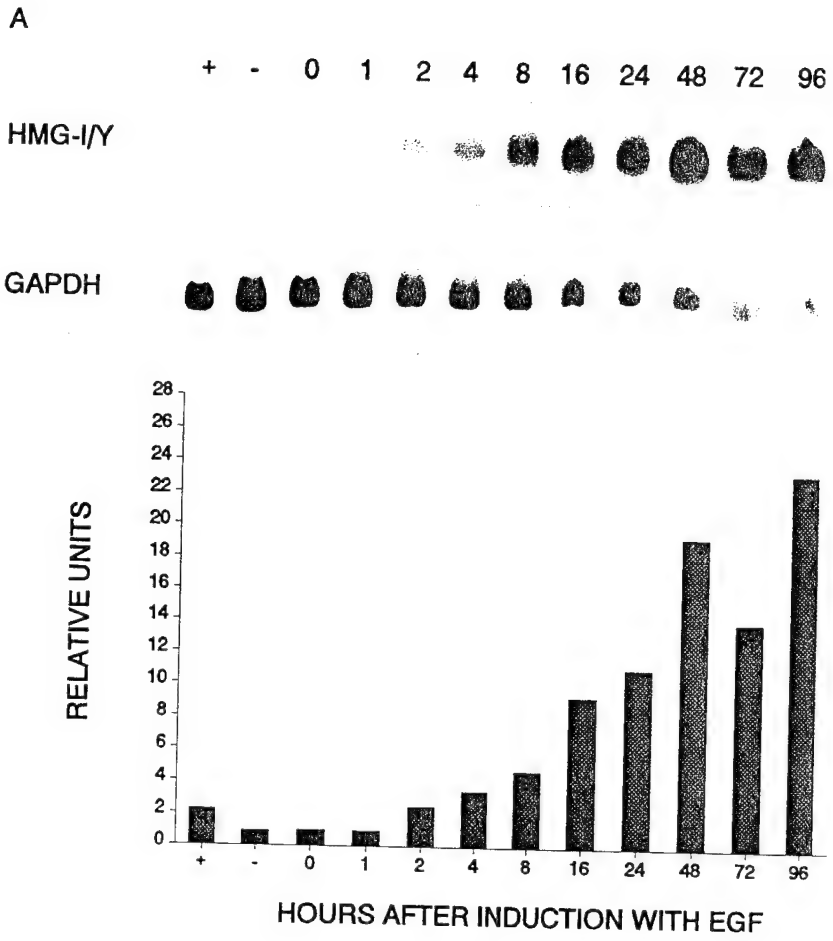

A
+ - 0 1 2 4 8 16 24 48 72 96
HMG-I/Y
GAPDH
RELATIVE UNITS
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
+ - 0 1 2 4 8 16 24 48 72 96
HOURS AFTER INDUCTION WITH EGF

FIGURE 2A

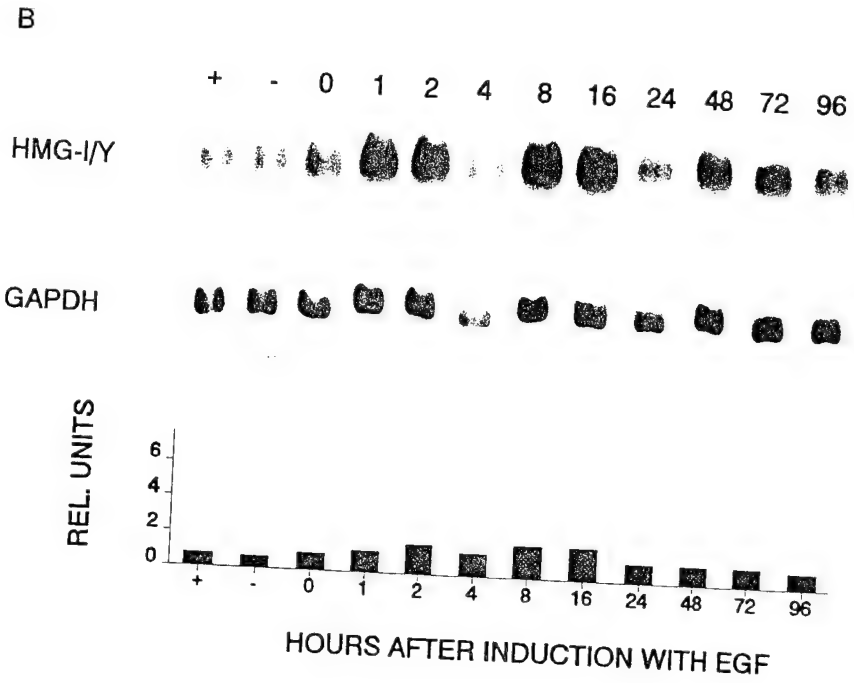

B
+ - 0 1 2 4 8 16 24 48 72 96
HMG-I/Y
GAPDH
REL. UNITS
6
4
2
0
+ - 0 1 2 4 8 16 24 48 72 96
HOURS AFTER INDUCTION WITH EGF

FIGURE 2B

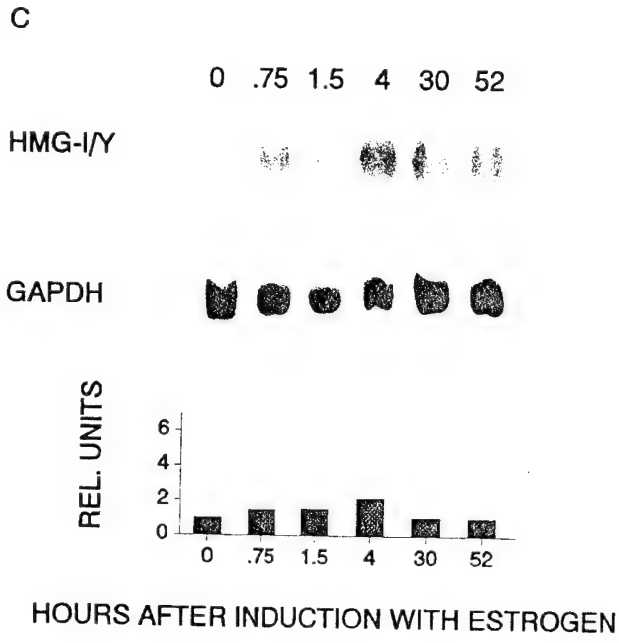

C
0 .75 1.5 4 30 52
HMG-I/Y
GAPDH
REL. UNITS
6
4
2
0
0 .75 1.5 4 30 52
HOURS AFTER INDUCTION WITH ESTROGEN

FIGURE 2C

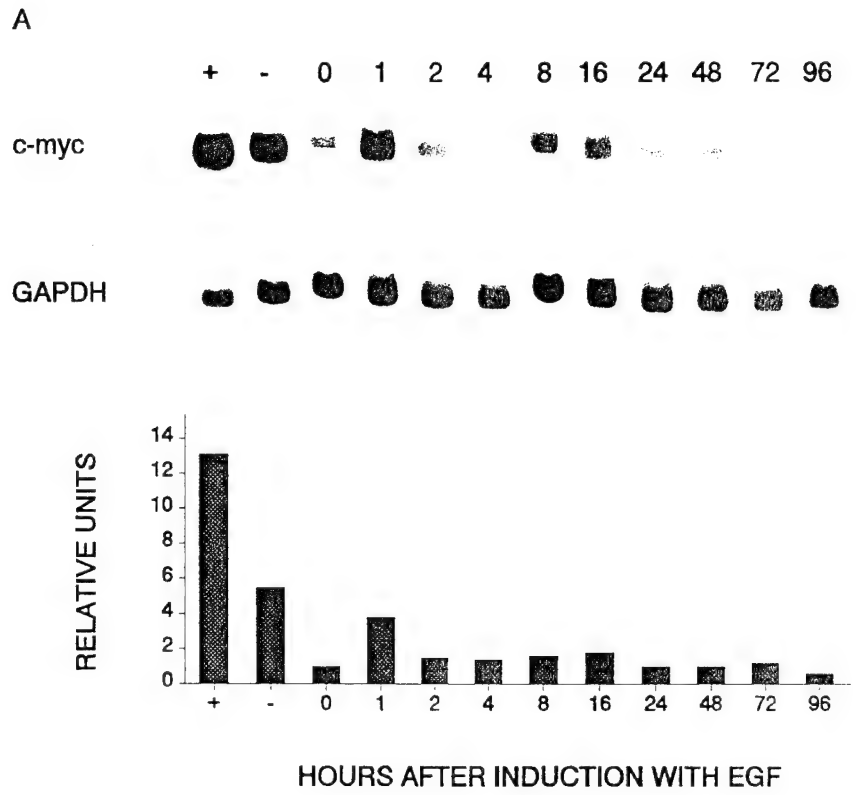

A
+ - 0 1 2 4 8 16 24 48 72 96
c-myc
GAPDH
RELATIVE UNITS
14
12
10
8
6
4
2
0
+ - 0 1 2 4 8 16 24 48 72 96
HOURS AFTER INDUCTION WITH EGF

FIGURE 3A

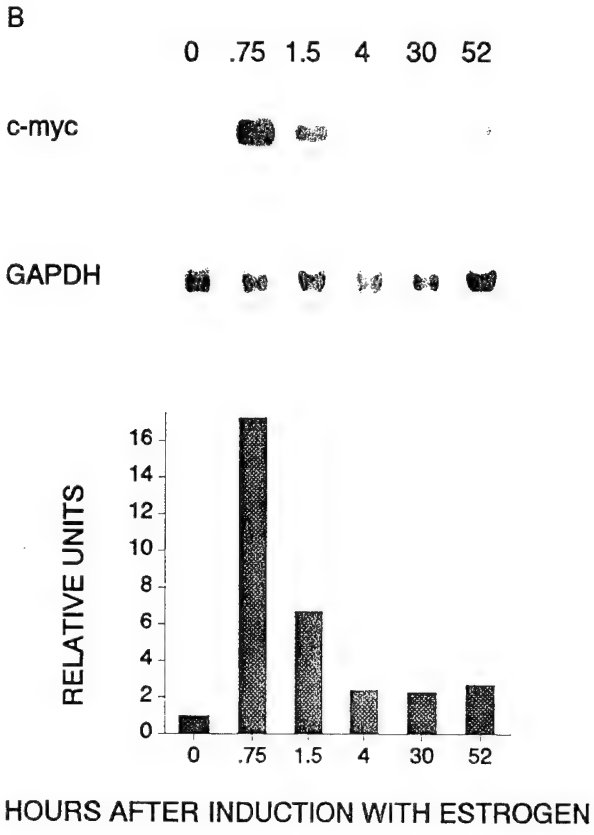

B
0 .75 1.5 4 30 52
c-myc
GAPDH
RELATIVE UNITS
16
14
12
10
8
6
4
2
0
0 .75 1.5 4 30 52
HOURS AFTER INDUCTION WITH ESTROGEN

FIGURE 3B

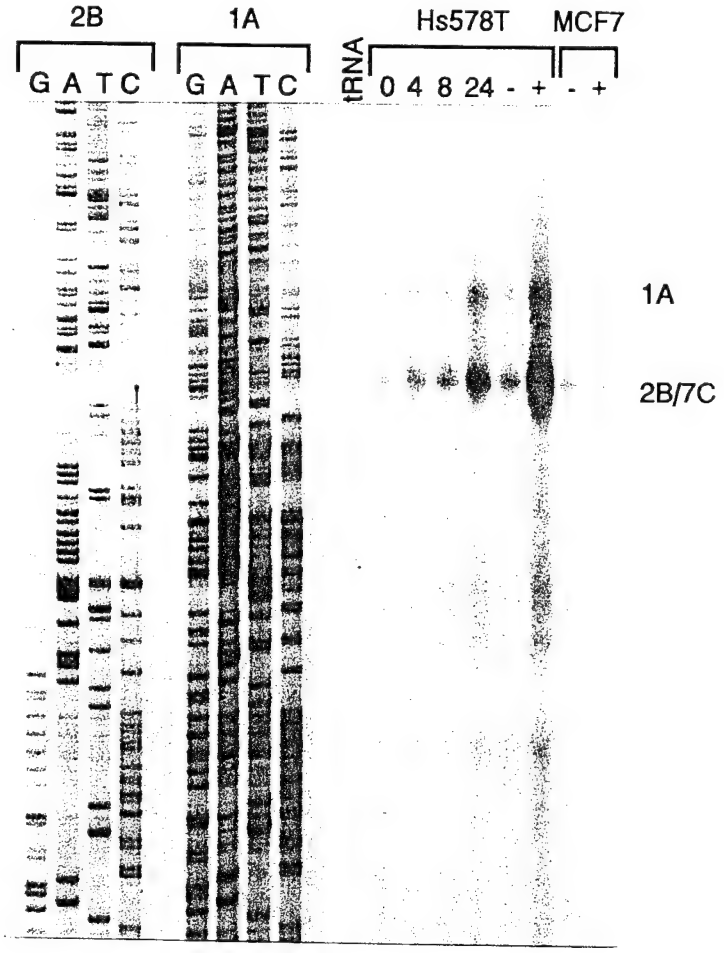


FIGURE 4

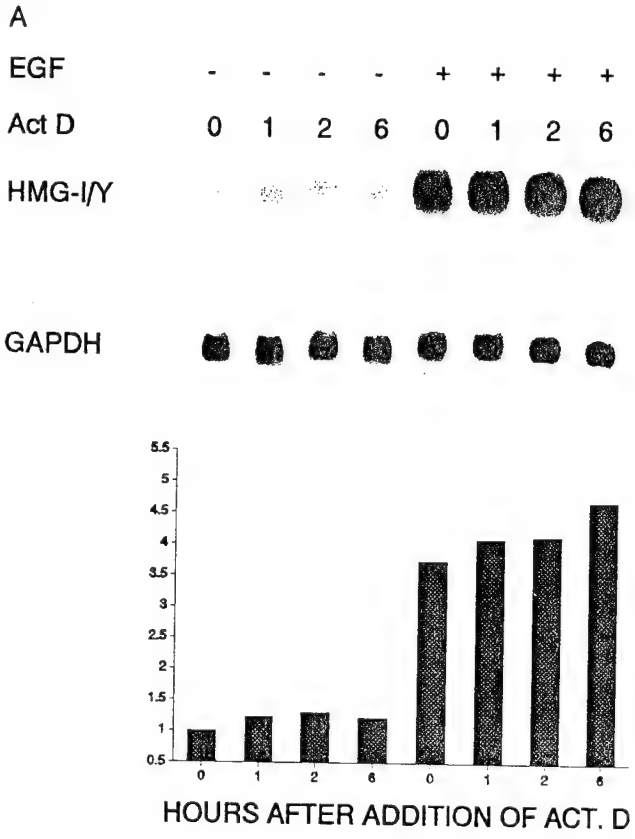

A
EGF
- - - - + + + +
Act D
0 1 2 6 0 1 2 6
HMG-I/Y
GAPDH
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0 1 2 6 0 1 2 6
HOURS AFTER ADDITION OF ACT. D

FIGURE 5A

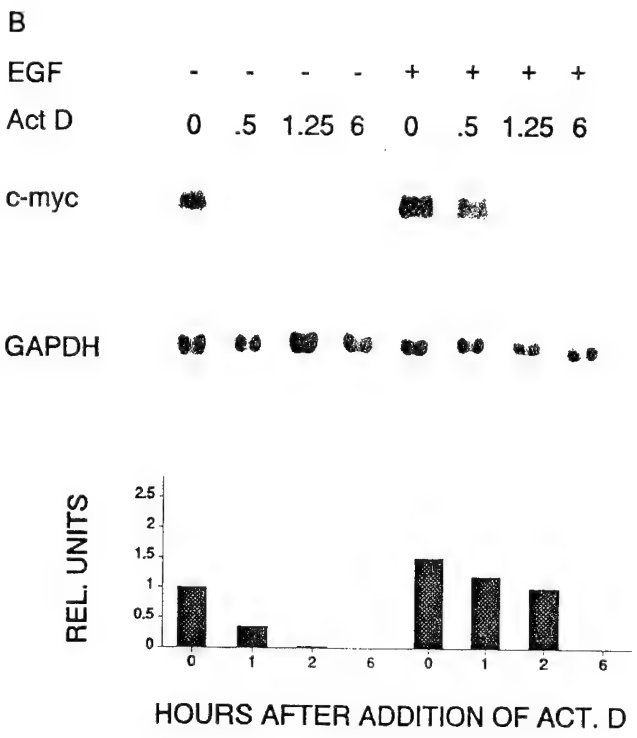
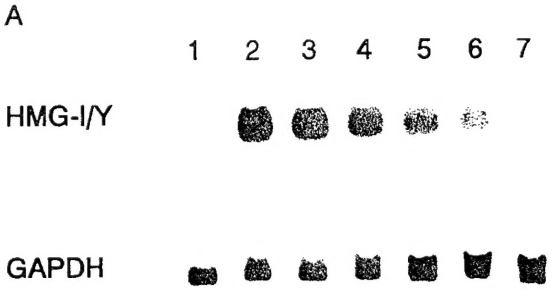

B
EGF
- - - - + + + +
Act D
0 .5 1.25 6 0 .5 1.25 6
c-myc
GAPDH
REL. UNITS
2.5
2
1.5
1
0.5
0
0 1 2 6 0 1 2 6
HOURS AFTER ADDITION OF ACT. D

FIGURE 5B

A



B

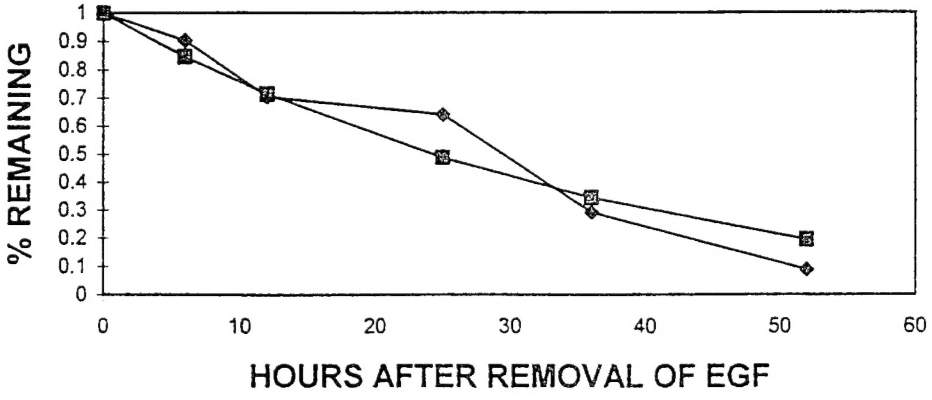
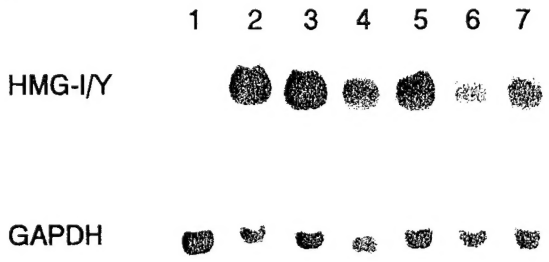


FIGURE 6A & 6B

C



D

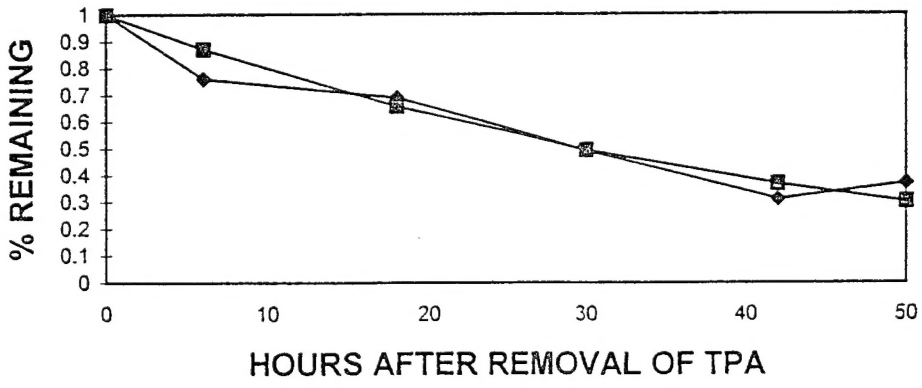


FIGURE 6C & 6D

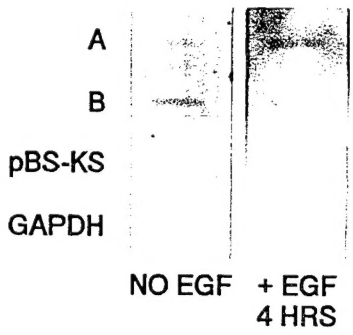


FIGURE 7

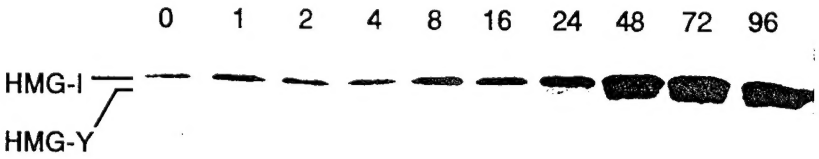


FIGURE 8